Purification and Properties of Acid Phosphatase in Bovine Milk

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The acid phosphatase of bovine milk has been purified over 40,000-fold by extensive use of Amberlite IRC-50. The enzyme is a basic protein with an optimum pH of 4.75. Ascorbic acid triples the activity, while other reducing agents have only a slight activating effect. The acid phosphatase acts on aromatic phosphates, casein, and pyrophosphates (both organic and inorganic). It did not act on AMP, serine phosphate, and glycerol phosphate.

INTRODUCTION

The acid phosphatase of bovine skim milk is not only low in amount, but the full activity is masked by inhibitory substances (1). The marked ability of Amberlite IRC-50[2] to adsorb acid phosphatase from milk provides an easy method for concentrating the enzyme free of inhibitory substances (2). The Amberlite IRC-50 removes from the milk all the acid phosphatase and only 0.5% of the total protein. Using this procedure as an initial step, further purification has been achieved and the purified enzyme has been studied.

MATERIALS

Unpasteurized skim milk was obtained from a local dairy. Twenty-five-gallon quantities were used for the isolation of acid phosphatase.

Amberlite CG-50 (chromatographic grade of IRC-50) was converted to the ammonium form using the method of Morrison *et al.* (3). The preparation of whole casein and calcium-sensitive α-casein from skim milk has been described by Zittle *et al.* (4).

METHODS

PROTEIN ESTIMATION

Protein concentrations were estimated as the optical density at 280 mμ using a Beckman DU spectrophotometer and a cell with a 1-cm. light path. A factor of 1 was used to convert Beckman readings to approximate protein concentration of mg./ml.

ACID PHOSPHATASE ACTIVITY

Acid phosphatase activity was estimated by Lowry's method (5) using disodium *p*-nitrophenyl phosphate (NPP)[3] as the substrate. For the standard test, 0.1 ml. of the appropriately diluted enzyme preparation was added to 0.9 ml. of the buffered substrate at 38°C., the final concentration of reactants being 0.005 *M* NPP and 0.10 *M* acetate buffer, pH 5.2. The reaction was stopped after an appropriate time interval (usually 5 min., although 30-min. intervals were required for unfractionated milk) by the addition of 3 ml. of 0.25 *N* NaOH. Free NP was estimated at 410 mμ. The unit of activity is defined as the amount of enzyme which liberates from NPP 1 μmole NP/min. under the above assay conditions. Specific activity is defined as the acid phosphatase units/mg. protein.

When substrates other than NPP were used, the acid phosphatase activity was measured by determining the phosphate released. The reaction was stopped by the addition of 3.3 ml. of 1.14 *N* H_2SO_4. The phosphate was determined by the

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[2] It is not implied that the U. S. Department of Agriculture recommends products of companies mentioned to the possible exclusion of others in the same business.

[3] The following abbreviations are used: NPP, disodium *p*-nitrophenyl phosphate; NP, *p*-nitrophenyl; ATP, adenosine triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-phosphate.

method of Sumner (6). The hydrolysis of NPP could be followed using both methods.

When casein was used as a substrate, it was necessary to precipitate the casein by the addition of 1 ml. of 20% trichloroacetic acid. Inorganic phosphate was then determined on the supernatant.

Starch-gel electrophoresis was done by the method of Smithies (7).

RESULTS

Fractionation Procedure

Step 1: Batchwise Adsorption to IRC-50

Unpasteurized skim milk obtained from a local dairy was conveniently processed in 25-gal. batches. The milk was stirred for 2 hr. with 1 kg. of Amberlite IRC-50 in the ammonium form. In subsequent experiments with 100-ml. samples of milk, adsorption occurred with a stirring time of 15 min. The resin was allowed to settle for 1 hr., and the milk was siphoned off. The resin was transferred to a 7-l. container and washed several times with distilled water. After each washing the water was removed by decantation.

The resin was eluted in three batches. A one-third portion of the resin was transferred to a 600-ml. sintered-glass funnel, and the resin was washed with water until the filtrate became clear. The acid phosphatase was eluted from the resin using 300-ml. portions of 1 *M* NaCl. The resin was stirred, and the NaCl eluate was collected in a filter flask. This elution was repeated twice.

The remaining washed resin was eluted in the same manner, and all the eluates were combined. It took less than 3 l. of 1 *M* NaCl to elute the acid phosphatase from 1 kg. resin. The specific activity of the acid phosphatase was 500 times greater than the specific activity of the acid phosphatase in milk. About 50% of this increase in specific activity was due to the separation of the phosphatase from the inhibitors in the milk, as the total acid phosphatase doubled in amount.

Step 2: Batchwise Adsorption to IRC-50, pH 9.0

The salt was removed from the eluate by dialysis for 3 days in the cold against 60 l. of distilled water. The water was changed once. The acid phosphatase of the dialyzed eluate (3100 ml., pH 6.35) was again adsorbed to the IRC-50 (40 g. in the ammonium form), and the pH of the mixture was adjusted to 9. The mixture was stirred for 2 hr., transferred to a sintered-glass funnel, and filtered. The resin was washed with copious amounts of distilled water. The acid phosphatase was eluted from the resin, using 200-ml. portions of 1 *M* NH_4Ac. Three elutions removed the bulk of the acid phosphatase. Total volume of the combined eluates was 540 ml. containing 0.7% protein.

Step 3: Acetone Precipitation

This step was carried out at 4°C. Acetone was added to the eluate until the concentration reached 46%. The cloudy suspension was centrifuged for 15 min. at 2000 r.p.m., and the precipitate was discarded. Acetone was added to the supernatant until the concentration reached 73%. After centrifuging for 15 min. at 2000 r.p.m., the green precipitate was dissolved in water. After this step the acid phosphatase had been concentrated 2000-fold.

Step 4: IRC-50 Chromatography

The acid phosphatase was equilibrated by dialysis in the cold against 0.2 *M* NH_4Ac for 15 hr. Some undissolved protein with no enzyme activity was removed from the dialyzed solution by centrifugation.

A 4.5 × 11 cm. column was prepared from Amberlite IRC-50 suspended in 0.2 *M* NH_4Ac. About 2 l. of 0.2 *M* NH_4Ac was allowed to flow through the column before the protein was added. The acid phosphatase solution containing 1.2 g. protein was placed on the column. Proteins were eluted using a concave gradient system, described by Bock and Ling [Ref. (8), Fig. 6]. The concentration of 920 ml. of 0.2 *M* NH_4Ac was changed by the gradual addition of 1,840 ml. of 1 *M* NH_4Ac. Figure 1 shows the results of a typical experiment. The initial peak, which contained the major part of the protein, contained both lactoperoxidase and ribonuclease. The acid phosphatase was eluted as two peaks—a large peak followed by a smaller one.

The pooled fractions from peak I (222 ml.) were dialyzed for 6 hr. against distilled

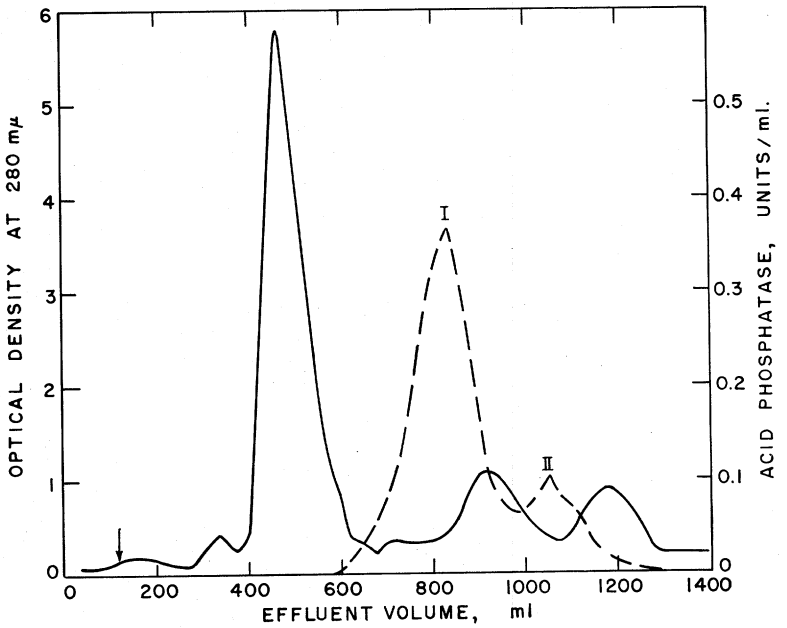

FIG. 1. Chromatogram of acid phosphatase (acetone-precipitated extract) on Amberlite IRC-50. Column size 4.5 × 11 cm. Flow rate 95 ml./hr. Buffer, 0.2 *M* NH_4Ac (pH 7.0). Arrow indicates start of gradient to 1.0 *M* NH_4Ac (pH 7.0). Solid line represents protein; dashed line represents acid phosphatase activity.

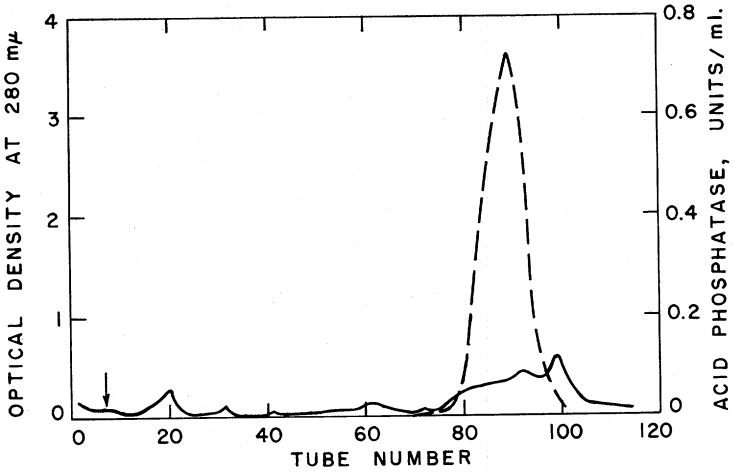

FIG. 2. Chromatogram of the acid phosphatase of peak I (Fig. 1) on Amberlite IRC-50. Column size 1 × 20 cm. Flow rate 60 ml./hr., 4 ml./tube. Buffer, 0.15 *M* NH_4Ac (pH 7.0). Arrow indicates start of gradient to 0.5 *M* NH_4Ac (pH 7.0). Solid line represents proteins; dashed line represents acid phosphatase activity.

water and reduced in volume by batchwise adsorption to 3.9 g. IRC-50 at pH 8.5. After stirring for 2 hr., the enzyme was eluted with 15 ml. of 1 *M* NH_4Ac (as previously described). The solution was dialyzed for 3 hr. against distilled water in the cold and overnight against 0.2 *M* NH_4·Ac.

Step 5: Rechromatography on IRC-50 Column

The acid phosphatase (19 ml.) was rechromatographed as shown in Fig. 2. A convex gradient [Ref. (8), Fig. 6] was produced using three containers (164 ml. solution in each) connected at the bottom with plastic tubing. The first two bottles contained 0.15 *M* NH_4Ac and the third bottle contained 0.5 *M* NH_4Ac. The acid phosphatase from tubes 80–98 was collected. The pooled fractions were lyophilized.

TABLE I
PURIFICATION OF ACID PHOSPHATASE

Fraction	Volume	Acid phosphatase	Protein	Specific activity
	ml.	*units*	*g.*	*units/mg.*
Milk	94,700	712	3,360	0.00021
1st IRC-50 eluate	2,760	1,457	14.5	0.106
2nd IRC-50 eluate	420	1,008	3.73	0.272
Acetone precipitate	75	580	1.28	0.454
IRC-50 chromatography (Peak I)	222	330	0.0963	3.43
Rechromatography on IRC-50	140	292	0.0304	9.72

A summary of a typical purification procedure is presented in Table I.

STARCH-GEL ELECTROPHORESIS

On starch-gel electrophoresis, the lyophilized preparation showed one broad protein band in Veronal buffer, pH 8.6. The protein was positively charged and traveled with a mobility approximately half that of crystalline pancreatic ribonuclease used as a reference protein. Acid phosphatase was determined on 5-mm. strips of the gel. The activity was located on the fastest moving end of the protein band.

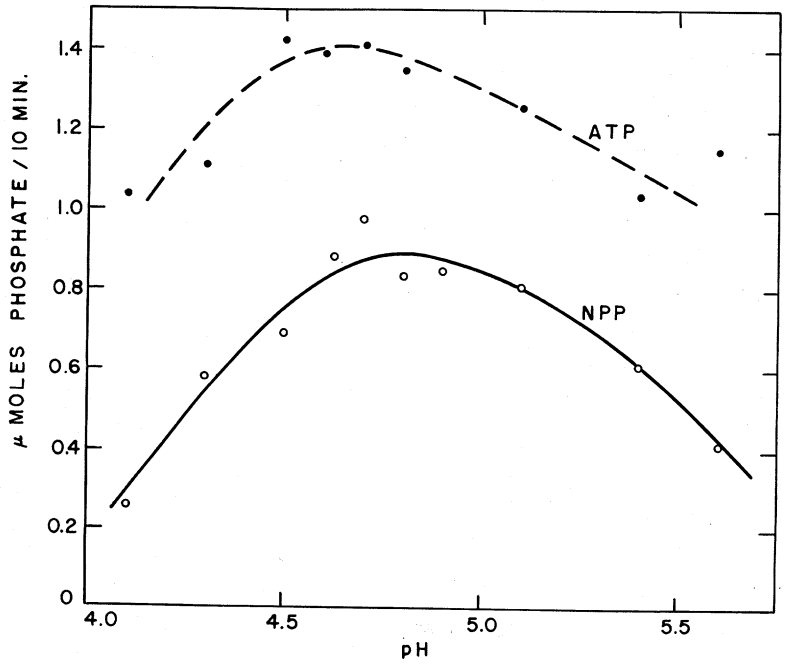

FIG. 3. Effect of pH on acid phosphatase, using ATP and NPP as substrates. Test system: 5 μmoles substrate, 100 μmoles acetate buffer, 10 μmoles neutralized ascorbic acid and 4 μg. enzyme in a final volume of 1 ml. incubated for 10 min. at 38°C.

TABLE II

ACTION OF ACID PHOSPHATASE ON PHOSPHATE ESTERS

Test system contained 5 μmoles substrate, 100 μmoles acetate buffer pH 4.75, 10 μmoles ascorbic acid (pH 4.75) and 4 μg. enzyme in a final volume of 1 ml., incubated for 10 min. at 38°C.

Substrate	Micromoles phosphate liberated
I. Phosphomonoesters	
A. Aromatic	
a. *p*-Nitrophenyl phosphate	1.184
b. Phenyl phosphate	0.512
c. *o*-Carboxylphenyl phosphate	0.962
B. Aliphatic	
a. AMP	0.019
b. *O*-Serine phosphate[a]	0
c. β-Glycerol phosphate[a]	0
II. Pyrophosphates	
a. ADP	1.20
b. ATP	0.829
c. Inorganic pyrophosphate	1.954
III. Polyphosphates	
a. Tetra[b]	0.357
b. Meta[b]	0.260
c. Hexametaphosphate[b]	0.368
d. Cyclo[b]	0–0.003
IV. Phosphoproteins	
a. Calcium-sensitive α-casein[b]	0.065
b. Whole casein[b]	0.029

[a] Reaction investigated at pH's 4.0, 4.8, and 5.7.

[b] Substrate contained 5 μmoles phosphorus.

PROPERTIES

pH Optimum

Optimal activity was obtained in the range from 4.6 to 4.8 as shown in Fig. 3. Previous studies using a crude milk preparation showed a broad pH optimum with little variation in the range pH 4.2–5.5 (1).

Substrate Specificity

The action of the enzyme preparation on a number of substrates is shown in Table II. The enzyme is very active toward compounds in which the phosphate group is esterified with a phenolic hydroxyl group. Aliphatic monoesters were not hydrolyzed.

Pyrophosphates (both organic and inorganic) were hydrolyzed rapidly, though the cyclic compound was not attacked.

Some hydrolysis of the phosphate group in casein occurred. The enzyme reaction was carried out at pH 5.8 as lower pH values precipitated some of the casein. At pH 5.8 the casein was hydrolyzed at approximately 10% of the rate of phenyl phosphate. In spite of this relatively low activity with casein, the acid phosphatase seems to have considerably greater phosphoprotein phosphatase activity than the alkaline phosphatase of milk, which hydrolyzes the casein phosphorus at only 0.5% of the rate of phenyl phosphate (9).

Activators

Acid phosphatase activity was increased by the addition of ascorbic acid to a variety of substrates. Results are shown in Table III. Ascorbic acid seemed unique in this respect as other reducing agents had only a slight effect. The effect of ascorbic acid on two substrates (ATP and NPP) is shown in Fig. 4.

TABLE III

EFFECT OF REDUCING AGENTS ON ENZYME ACTIVITY WITH DIFFERENT SUBSTRATES

Test system contained 10 μmoles reducing agent, 100 μmoles acetate buffer of pH 4.75, 5 μmoles substrate and 4 μg. enzyme in a final volume of 1 ml. incubated for 10 min. at 38°C.

Reducing agent	Substrate			
	NPP	ATP	Inorganic pyrophosphate	Casein phosphate
	% activation			
Ascorbic acid	300	316	140	364
2-Mercaptoethanol	11			
l-Cysteine	22	61		
Reduced glutathione	12			
N-Ethylmaleimide	0			
$FeSO_4$ (2 μmoles)	35			
Thioglycolate		37		

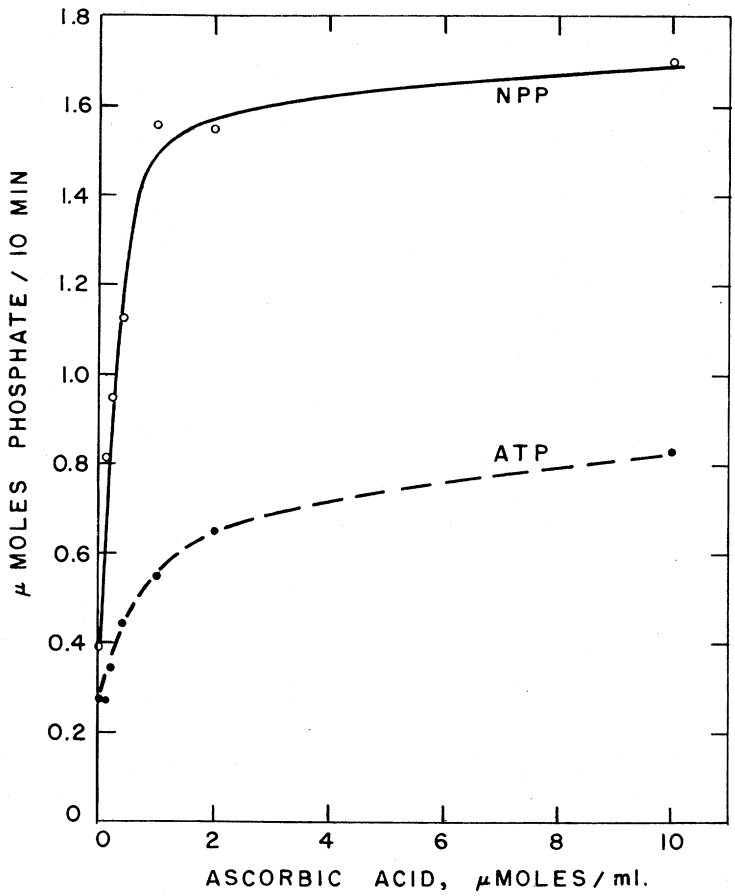

FIG. 4. Effect of ascorbic acid concentration on enzyme activity. Test system is described in legend for Fig. 3; pH 4.8. Ascorbic acid concentration varied.

DISCUSSION

The acid phosphatase of skim milk bears some resemblance to the phosphoprotein phosphatase of bovine spleen (10–13). Qualitatively, both enzymes are active with the same type of substrates, are activated by ascorbic acid, and are highly basic proteins. Certain differences are evident. First, the milk enzyme showed very little activation with thioglycolate, cysteine, mercaptoethanol and *N*-ethylmaleimide, all of which more than doubled the activity of the spleen phosphoprotein phosphatase. Secondly, ascorbic acid inactivates the spleen phosphoprotein phosphatase when preincubated with the enzyme. However, the milk enzyme could not be inactivated by ascorbic acid.[4] Thirdly the milk phosphatase has relatively low activity against casein, compared to other substrates.

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